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The ceramide activated protein phosphatase Sit4 impairs sphingolipid dynamics, mitochondrial function and lifespan in a yeast model of Niemann-Pick type C1

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ABSTRACT

The Niemann-Pick type C is a rare neurodegenerative disease that results from loss-of-function point mutations in *NPC1* or *NPC2*, which affect the homeostasis of sphingolipids and sterols in human cells. We have previously shown that yeast lacking Ncr1, the orthologue of human NPC1 protein, display a premature ageing phenotype and higher sensitivity to oxidative stress associated with mitochondrial dysfunctions and accumulation of long chain bases. In this study, a lipidomic analysis revealed specific changes in the levels of ceramide species in *ncr1*Δ cells, including decreases in dihydroceramides and increases in phytoceramides. Moreover, the activation of Sit4, a ceramide-activated protein phosphatase, increased in *ncr1*Δ cells. Deletion of *SIT4* or *CDC55*, its regulatory subunit, increased the chronological lifespan and hydrogen peroxide resistance of *ncr1*Δ cells and suppressed its mitochondrial defects. Notably, Sch9 and Pkh1-mediated phosphorylation of Sch9 decreased significantly in *ncr1*Δ*sit4*Δ cells. These results suggest that phytoceramide accumulation and Sit4-dependent signaling mediate the mitochondrial dysfunction and shortened lifespan in the yeast model of Niemann-Pick type C1, in part through modulation of the Pkh1-Sch9 pathway.

1. Introduction

Sphingolipids are important structural components of cell membranes, highly conserved among species, and their bioactive metabolites can act as signaling molecules regulating many biological processes. Sphingosine, a long chain sphingoid base (LCB), and ceramide are involved in the regulation of actin cytoskeleton organization, endocytosis, apoptosis, cell senescence and cell cycle arrest whereas sphingosine-1-phosphate plays a key role in proliferation, mitogenesis, cell migration, cell survival and inflammation (reviewed in [1,2]). The homeostasis of sphingolipids is affected in several age-associated neurological diseases, such as Alzheimer's [3] and Parkinson's [4] diseases, in cancer [5], as well as in sphingolipidoses, a group of lysosomal storage diseases that comprise several distinct defects in lysosomal enzymes and lipid transfer proteins [6]. A characteristic feature of the sphingolipidoses is the accumulation of other secondary storage products because of the lipid nature of the primary storage compound, leading to a traffic jam [7].

Niemann-Pick type C (NPC) is a sphingolipidosis caused by loss-of-function point mutations in *NPC1* or *NPC2* gene [8,9] and is clinically characterized by a severe neurodegeneration and accentuated failure of systemic organs such as liver and spleen. The NPC1 protein is a large transmembrane protein that is located in the transient late endosome/lysosome system, while NPC2 protein is a soluble glycoprotein with high affinity for cholesterol [10,11]. Both proteins seem to be involved in intracellular transport of endocytosed cholesterol through the endolysosomal system [12]. In addition to cholesterol accumulation concomitant with sphingomyelin and gangliosides storage [13], there is an increase in the levels of sphingosine that precedes cholesterol entrapment in the lysosome of NPC1 cells [14].

Abbreviations: CAPP, ceramide-activated protein phosphatase; CLS, chronological lifespan; COX, cytochrome *c* oxidase; dhCer, dihydroceramide; DHS, dihydrosphingosine; LCB, long chain sphingoid base; MAPK, mitogen activated protein kinase; NPC, Niemann-Pick type C; PDS, post-diauxic shift; phytoCer, phytoceramide; PHS, phytosphingosine; ROS, reactive oxygen species

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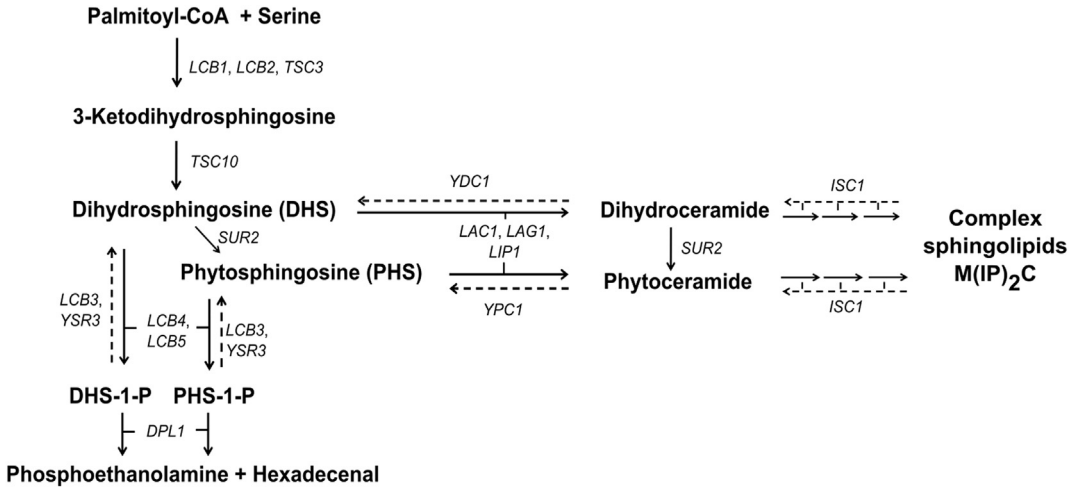

Fig. 1. Schematic representation of sphingolipid metabolism in yeast. Metabolic intermediates and genes encoding the most important enzymes involved in each step of sphingolipid synthesis and turnover are shown.

Sphingolipid metabolism is highly conserved from yeast to mammalian and has been extensively studied using this simple model organism (Fig. 1) [15]. We have recently reported that yeast cells lacking Ncr1, an orthologue of human NPC1 [16], exhibit high levels of sphingosine and hyperactivation of the Pkh1-Sch9 pathway that are associated with severe mitochondrial dysfunctions, oxidative stress sensitivity and premature ageing [17]. Several studies suggest that sphingolipid homeostasis plays an important role in oxidative stress resistance and longevity in yeast, as also described in mammals [18,19]. Lag1, a component of ceramide synthase, regulates replicative lifespan [20,21]. Yeast cells lacking Ydc1, a dihydroceramidase that hydrolyzes dihydroceramide into the LCB dihydrosphingosine, exhibit an increased chronological lifespan (CLS) whereas cells overexpressing *YDC1* show mitochondria and vacuolar fragmentation, increased apoptosis and a shortened CLS [22]. The levels of LCBs increase in stationary phase cells due to a decrease in the activity of both ceramide synthase (Lag1) and LCB kinase (Lcb4) [23], and the down-regulation of sphingolipid synthesis extends CLS in part due to a reduction in LCB-mediated activation of Sch9 [24], the yeast homologue of mammalian ribosomal protein S6kinase (S6K) also related to Akt/protein kinase B [25,26]. Moreover, yeast lacking Isc1, an orthologue of mammalian neutral sphingomyelinase-2 (nSMase2) that generates ceramide through the hydrolysis of inositol phosphosphingolipids, display a shortened CLS, oxidative stress sensitivity, mitochondrial dysfunction, iron overload and caspase-dependent apoptosis [27].

Defects in NPC1 function result in the accumulation of ceramide in the liver of NPC1 patients [28]. In addition, the deficiency of a NPC1-related protein in the intracellular parasite *Toxoplasma* increases the accumulation of cholesteryl esters, sphingomyelin, as well as of ceramide [29]. In mammals, changes in ceramide levels have been implicated in apoptosis through the modulation of signaling proteins [30,31], including protein kinase C (PKC), cathepsin D, JNK, ceramide-activated protein kinases and ceramide-activated protein phosphatases (CAPPs) [32–35]. The CAPPs are found in all eukaryotes and are composed by one catalytic subunit and two regulatory subunits [36]. The yeast CAPP is constituted by the Tpd3 and Cdc55 regulatory subunits and by the Sit4 catalytic subunit [37,38]. Sit4 is a serine-threonine protein phosphatase with high homology to human protein phosphatase 6 [39]. It has been implicated in the regulation of the cell cycle [40], mitochondrial function [41–43], carbohydrate metabolism [44,45], homeostasis of monovalent ion and pH [46], the Pkc1-MAPK pathway [47] and traffic from the ER to the Golgi complex [48]. Moreover, Sit4 is down regulated by the Target of Rapamycin Complex 1 (TORC1) [49,50]. Interestingly, a recent study implicated NPC1 in mTORC1 regulation in response to lysosomal cholesterol. Castellano et al. showed that lysosomal cholesterol drives mTORC1 recruitment to lysosomes and activation. Moreover, NPC1 mediates mTORC1 inhibition upon cholesterol depletion and mTORC1 is constitutively active in NPC1 deleted cells [51].

Our laboratory has shown that *SIT4* deletion increases CLS and H_2O_2 resistance through modulation of mitochondrial function [52]. Moreover, we reported that Hog1 MAPK is a downstream effector of Sit4 that regulates mitochondrial function, being activated in response to ceramide by a Sch9-dependent mechanism [53,54]. Both Sch9 and Sit4 are also modulators of sphingolipid metabolism [55,56]. These results support a functional cross talk between Sit4 and Sch9 in response to ceramide changes.

In this report, we show that the activation of Sit4/CAPP associated with an increase in the levels of long chain phytoceramide species impairs oxidative stress resistance, mitochondrial function and chronological lifespan in *ncr1*Δ cells. Notably, the suppression of *ncr1*Δ phenotypes by *SIT4* deletion is associated with downregulation of the Pkh1-Sch9 pathway.

2. Materials and methods

2.1. Yeast strains and growth conditions

The *Saccharomyces cerevisiae* strains used in this work are listed in table 1. The growth media used were YPD [1% (w/v) yeast extract, 2% (w/v) bactopeptone, 2% (w/v) glucose], YPG [1% (w/v) yeast extract, 2% (w/v) bactopeptone, 2% (v/v) glycerol], synthetic complete (SC) drop-out medium containing 2% (w/v) glucose 0.67% yeast nitrogen base without amino acids or minimal medium containing 2% (w/v) glucose 0.67% yeast nitrogen base without amino acids supplemented with appropriate amino acids [0.008% (w/v) histidine, 0.04% (w/v) leucine, 0.008% (w/v) tryptophan)] and 0.008% (w/v) uracil. Yeast cells were grown aerobically at 26 °C in an orbital shaker (at 140 rpm), with a ratio of flask volume:medium volume of 5:1, to early exponential phase (OD_{600nm} = 0.6) or to post-diauxic shift phase (PDS; OD_{600nm} = 7–9).

Deletion strains were generated using PCR-derived deletion cassettes containing *URA3*, *HIS3* or *KanMX4* with flanking regions of each gene. Yeast cells were transformed using the lithium acetate/ single-stranded DNA/ polyethylene glycol protocol [57].

To evaluate the levels of Lag1 and Ypc1 expression, *ncr1*Δ *LAG1-9MYC* and *ncr1*Δ *YPC1-9MYC* cells were generated by disruption of the *NCR1* gene in BY4741 cells with *LAG1* and *YPC1* genomically tagged with 9Myc, respectively [55] All the disruptions were confirmed by standard PCR procedures.